Polymorphism in a nitric oxide synthase gene

Background of the Invention

Field of the Invention

This invention relates to a polymorphism in a nitric oxide synthase (NOS) gene and to diagnostic method and apparatus based upon the polymorphism, in particular a polymorphism that is located within the promoter region of the gene. The invention also relates to methods of identifying individuals having a predisposition or susceptibility to essential hypertension and also to the group of conditions that contribute to Syndrome X and to methods of treating those individuals to prevent, delay or reduce disease.

Related Art

Essential hypertension is a common multifactorial disorder that affects approximately 20% of Caucasian adults. It results in a significantly increased risk for heart attack and stroke. The condition has a genetic basis, although at present the number of genes is unknown. Hypertension is also known to cluster with obesity and other disorders such as non-insulin dependent diabetes (NIDDM), atherosclerosis, vascular disease and dyslipidaemia in a metabolic syndrome known as Syndrome X. If the genes that cause susceptibility to Syndrome X disorders can be identified, then treatments for specific gene defects can be targeted.

Inducible NOS (iNOS) is expressed in a variety of tissues including myocytes, small vessel endothelium, vascular smooth muscle cells, hepatocytes, renal proximal tubule, Henle's loop, macula densa, afferent arteriol, astrocytes and immune cells17. Nitric oxide, produced by either constitutive or inducible isoforms of NOS, influences smooth muscle vasodilation suggesting that it may

play a role in regulating blood pressure18. However, no genetic link between NOS genotype and hypertension has so far been identified.

Three members of the iNOS II gene family NOS2A, NOS2B and NOS2C have been co-localized to human chromosome 17 between bands p13.1 and q255. Furthermore, the human NOS2A gene, localised to 17cen - q11.214, contains a biallelic 4 bp repeat polymorphism located within the promoter13.

At present, the only available treatments for hypertensive disorders are pharmaceutical based medications that are not targeted to an individual's actual defect; examples include ACE inhibitors and diuretics for hypertension, insulin supplementation for NIDDM, cholesterol reduction strategies for dyslipidaemia, anti-coagulants, β blockers for cardiovascular disorders and weight reduction strategies for obesity. If targeted treatment strategies were available it might be possible to predict the response to a particular regime of therapy and could markedly increase the effectiveness of such treatment. Although targeted therapy requires accurate diagnostic tests for disease susceptibility, once these tests are developed the opportunity to utilise targeted therapy will become widespread. Such diagnostic tests could initially serve to identify individuals at most risk of hypertension and could allow them to make changes in lifestyle or diet that would serve as preventative measures. The benefits associated by coupling the diagnostic tests with a system of targeted therapy could include the reduction in dosage of administered drugs and thus the amount of unpleasant side effects suffered by an individual. In more severe cases a diagnostic test may suggest that earlier surgical intervention would be useful in preventing a further deterioration in condition.

Summary of the Invention

It is an object of the invention to provide genetic diagnosis of predisposition or susceptibility to Syndrome X, and to hypertension in particular. Another related object is to provide treatment to reduce or prevent or delay the onset of disease in those predisposed or susceptible to this disease. A further object is to provide means for carrying out this diagnosis.

Accordingly, a first aspect of the invention provides a method of diagnosis of disease in an individual, said method comprising determining the genotype of a NOS gene in said individual.

In another aspect, the invention provides a method of identifying an individual predisposed or susceptible to a disease, said method comprising determining the genotype of a NOS gene in said individual.

The invention is of advantage in that it enables diagnosis of a disease or of certain disease states via genetic analysis which can yield useable results before onset of disease symptoms, or before onset of severe symptoms. The invention is further of advantage in that it enables diagnosis of predisposition or susceptibility to a disease or of certain disease states via genetic analysis.

The invention may also be of use in confirming or corroborating the results of other diagnostic methods. The diagnosis of the invention may thus suitably be used either as an isolated technique or in combination with other methods and apparatus for diagnosis, in which latter case the invention provides a further test on which a diagnosis may be assessed.

Detailed Description of the Preferred Embodiments

The present invention stems from using allelic association as a method for genotyping individuals; allowing the investigation of the molecular genetic basis of essential hypertension. In a specific embodiment the invention tests for the presence of a 4 base pair insertion in a repeat sequence within the promoter of the NOS2A gene. The invention demonstrates a link between this tetranucleotide insertion and predisposition to essential hypertension by showing that an increased number of hypertensives when compared to normotensives possess the NOS2A 4 base pair promoter insertion.

Certain disease states would benefit, that is to say the suffering of the patient may be reduced or prevented or delayed, by administration of treatment or therapy in advance of disease appearance; this can be more reliably carried out if advance diagnosis of predisposition or susceptibility to disease can be diagnosed.

In a particular embodiment of the invention, the method comprises determining genotype of a repeat region located 5' to the coding sequence within the promoter of an iNOS gene. The polymorphism is preferably a four base pair insertion located in the region -891 to -575 base pairs 5' to the transcription start site. The present invention in a specific example describes the location of a hypertension susceptibility locus on chromosome 17 and specifically implicates the inducible nitric oxide synthase gene, NOS2A, at 17cen-q11.2. Accordingly the invention provides strong evidence for a chromosome 17 role in human essential hypertension.

The method of the invention optionally comprises determining whether an individual is homozygous or heterozygous for polymorphisms of said iNOS gene. A determination that an individual is free of a risk genotype may provide a more

significant diagnosis. Likewise, presence of two risk alleles may give a significant diagnosis of predisposition to disease, and particularly hypertension.

In an embodiment of the invention, the disease is Syndrome X. The invention thus assists in identifying those individuals predisposed or susceptible to this syndrome, enabling early commencement of therapy or treatment or other techniques to avoid or reduce the disease, these latter including adopting a different lifestyle or a different diet. A number of individual disorders are known to be contained within or typically contribute to or feature in Syndrome X and references to Syndrome X are intended to be references to one or more diseases selected from the group consisting of hypertension, obesity, non-insulin dependent diabetes, atherosclerosis, dyslipidaemia, vascular and coronary artery disease.

It is therefore a further aspect of the invention to provide a method of treatment of an individual comprising determining genotype of a promoter region of an iNOS gene, determining if that individual is predisposed or susceptible to Syndrome X and if that individual is so diagnosed providing treatment to reduce or delay or prevent disease.

Current treatments and therapies for Syndrome X are all of application in the present invention for treatment and therapy for an individual diagnosed as predisposed or susceptible to Syndrome X. Insulin supplements are suitable for non-insulin dependent diabetes. A strategy to reduce cholesterol intake is suitable for dyslipidaemia. Anti-coagulants and β-blockers are suitable for cardiovascular disorders. Weight reduction strategy is suitable for obesity.

In a specific embodiment of the invention there is provided diagnosis of predisposition or susceptibility to hypertension. Suitable hypertension treatments are disclosed in US-A-5510390, 5496569, 5405872 and 5409936, the contents of which are incorporated herein by reference. Accordingly, the method may further comprise a treatment selected from the group consisting of administration of an

effective amount of antihypertensive pharmaceutical, administration of an effective anti-hypertension therapy or administration of both an effective anti-hypertension therapy and an effective amount of antihypertensive pharmaceutical.

Anti-hypertension therapy may include correction of obesity, high alcohol intake, high salt intake and/or lack of regular exercise. Anti-hypertensive pharmaceuticals may include beta-adrenoceptor blocking drugs, optionally in combination with a thiazide, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors, vasodilators, alpha-blockers and centrally acting drugs such as prazosin, terazosin and doxazosin. One embodiment of the present invention is that a particular polymorphism may indicate that a certain course of treatment could meet with a greater level of success than other treatments. Thus an advantage of the present invention is that it could assist in the choice of which of the many available therapies should be administered to the patient.

Determination of the genotype of said iNOS gene is suitably accomplished by screening the promoter region of said iNOS gene to identify a polymorphism in said 5' region of said iNOS gene, said polymorphism being indicative of a risk genotype in said individual. In an embodiment of the invention, the screening is accomplished by a technique selected from the group of techniques consisting of amplification of a nucleic acid sequence located in said 5' region of the iNOS gene, Southern Blotting of said 5' region of the iNOS gene and single strand conformational polymorphism (SSCP) mapping of said 5' region of the iNOS gene. The invention also encompasses screening the whole or a part of the promoter region of an iNOS gene for a polymorphism in linkage disequilibrium with a polymorphism in or near the 5' region of the iNOS gene.

The invention further encompasses the identification of other polymorphisms that are correlated with a known polymorphism in or near the 5' promoter region of an iNOS gene consisting of:-

(a) locating a polymorphism and correlating it with the known NOS gene polymorphism; and

(b) testing whether the new polymorphism is linked to Syndrome X or any contributory component thereof.

At present there is one other known polymorphism in the NOS2A promoter, located between 2.7 and 2.5 kb upstream of the transcription start site31. This polymorphism consists of a CCTTT pentanucleotide repeat and has a heterozygosity of 80.2%. In humans the number of these CCTTT repeats present in the promoter can vary from 9 to 16. Any effect of this particular polymorphism on transcription of the NOS2A gene is yet to be confirmed and it may therefore be linked with a predisposition to essential hypertension.

A specific example of the invention described in more detail below, uses one or more primers which will, following conventional polymerase chain reaction (PCR) techniques, amplify a nucleic acid sequence located in said promoter region of the NOS2A gene in particular between positions -891 and -575 base pairs 5' to the transcription start site. The product of the PCR includes an amplified nucleic acid sequence (SEQ ID NO:1). The next step is to determine the size of the amplified sequence. A suitable method is capillary electrophoresis, in which nucleic acids of different sizes migrate in a medium, typically a gel, at a rate according to their size. Two particular PCR primers (ref. 13) have a nucleotide sequence selected from the group of nucleotide sequences consisting of SEQ ID NO:2 and SEQ ID NO:3, though other primers may be used for this purpose.

SEQ ID NO:2

5' TGGTGCATGCCTGTAGTCC

SEQ ID NO:3

5' GAGGCCTCTGAGATGTTGGTC

These two primers are adapted to amplify a nucleic acid sequence located within said promoter region of the NOS2A gene. A risk genotype incorporates a four base insertion allele of 317 bp in size, a non-risk genotype incorporates a wild type allele of 313 bp in size, and the diagnosis of the invention may be carried out in particular on a human.

The invention also provides use of means to determine genotype of a promoter region of an iNOS gene in manufacture of apparatus for diagnosis of predisposition or susceptibility to Syndrome X. In an embodiment of this aspect of the invention, the PCR primers are adapted to amplify a fragment located within said promoter region of said gene, which region consists of or comprises positions -891 and -575 base pairs 5' to the transcription start site.

The invention still further provides a kit for diagnosis of predisposition or susceptibility to Syndrome X comprising one or more primer nucleic acid molecules for determining genotype of promoter region of an iNOS gene and apparatus for correlating iNOS genotype with risk of predisposition or susceptibility to disease. In a specific embodiment of the invention said apparatus for correlating iNOS genotype with risk comprises a set of reference markers that are run on a gel alongside the products of the PCR reaction. In another specific embodiment of the invention said apparatus comprises a set of reference gels that are compared to the gel on which the PCR products have been run thus allowing determination of a risk genotype. In a further specific embodiment of the invention said apparatus comprises a chart on which is indicated the size of the PCR products that allow identification of a risk genotype.

A preferred kit of the invention comprises PCR primers adapted to distinguish between risk and non-risk genotypes of a promoter region of an iNOS gene. Particularly preferred is one comprising primers adapted for amplification of the whole or a fragment of a region consisting of or comprising positions -891

and -575 base pairs 5' to the transcription start site, such as primers SEQ ID NO:2 and SEQ ID NO:3.

According to the present invention, there is a significant association of the tested polymorphism marker in the Syndrome X disease hypertension. There now follows a brief description of particular embodiments of the invention.

Examples

The NOS2A tetranucleotide repeat polymorphism was tested for linkage in 177 hypertensive sibpairs and for allelic association in 77 hypertensive and 76 normotensive individuals. SPLINK results indicated significant excess allele sharing with the biallelic NOS2A polymorphism (P = 0.0002). ASPEX15,16, a recently released analysis package, which uses an alternate restriction to SPLINK when performing maximum likelihood calculations, was also used to analyze NOS2A linkage data. Results using ASPEX indicated significant excess allele sharing and linkage of NOS2A in our hypertensive sibpair population (MLOD = 4.4). In addition, allelic association, as tested by chi-square analysis, indicated a significant association of the NOS2A polymorphism with hypertension ($\chi 2 = 5.9$; P = 0.016). As shown in Table 2, an increased number of hypertensives (19%) compared to normotensives (9%) possessed the NOS2A 4 bp promoter insertion. The odds ratio for hypertension associated with this insertion was estimated to be 2.3 (95% CI = 1.1-4.8).

Subjects. Blood from 239 Caucasian hypertensive siblings (blood pressure ≥ 140/90 mmHg prior to anti-hypertensive medication) was collected from contacts obtained through the National Health and Medical Research Council of Australia (NHMRC) Twin Registry and also from general practitioners and media releases. In addition, for the allelic association studies, blood was collected from 77 hypertensives (blood pressure ≥ 140/90 mmHg prior to medication and who were the offspring of two hypertensive parents) and from 76 normotensives (blood

pressure < 140/90 mmHg and who were the offspring of two normotensive parents), as previously described20. A detailed questionnaire was completed by all participants to obtain demographic parameters, to determine ancestry and to exclude those with a family history of diabetes and thyroid disease.

Genotyping. Genomic DNA was extracted from blood samples and markers genotyped using PCR and capillary electrophoresis, as previously described21. Fluorescently labelled primers were used to amplify DNA to detect the NOS2A biallelic marker13. All PCR products were genotyped using an ABI PRISM 310 Genetic Analyzer with GeneScan Software (Applied Biosystems, Foster City, CA).

Statistical analysis. Genotypes for the affected sibpairs were assessed and analyzed for linkage using both identity by state (IBS) and identity by descent (IBD) nonparametric methods. The extent of allele sharing was determined using the affected pedigree member (APM)9,10, SPLINK8,9 and ASPEX15,16 statistical packages. For APM analysis, maximum-likelihood estimates of allele frequencies for the 13 markers used in the chromosome 17 scan, were calculated from hypertensive sibship data, using the USERM13 program25 of the MENDEL package of programs26. For SPLINK and ASPEX analysis, maximum-likelihood estimates of the allele frequencies were internally calculated. Maximization of the likelihood ratio for SPLINK analysis was restricted to the possible triangle restriction (ie. $z[1] < 0.5$ and $z[0] < 0.5 \times z[1]$) (refs. 7,8). Additionally, to allow for a moderate amount of genetic dominance variance, maximum likelihood calculations for ASPEX (aspex.phase) analysis of NOS2A linkage data, assumed the following multiplicative model for z values: $z[2] = y2$, $z[1] = 2y(1-y)$ and $z[0] = (1-y)2$, where y is the sharing at this locus, derived using a moderate sibling recurrence risk ratio (λs) of 1.6 (refs. 15,16). In hypertension, there is some suggestive evidence for modest dominance variance due to higher correlation values for systolic and diastolic blood pressure between pairs of siblings than between pairs of parent and offspring (genetic dominance variance estimated at

0.18 and 0.22 for systolic and diastolic blood pressure, respectively)27. Allelic association results for NOS2A were analyzed by the chi-square test. Odds ratio and 95% confidence limit calculations were performed using the Epi Info Version 6 statistical program28.

Table 1 Relevant characteristics of hypertensive siblings

Category	n	Measurement
Pre-treatment systolic BP (mmHg)	99	169.4 ± 24.0
Pre-treatment diastolic BP (mmHg)	99	102.2 ± 10.2
BMI (kg/m2)	210	27.2 ± 5.2
Age (years)	227	55 ± 11
Siblings - male	84	
- female	155	
⇒ total	239	
Sibpairs - male : male	23	
- female : female	79	
- female : male	75	
⇒ total	177	

Data are mean ± standard deviation; BP = blood pressure; BMI = body mass index

Table 2 Association analysis of NOS2A in hypertensive and normotensive subjects

		Genotypes (bp)			Alleles (bp)	
Population	n	313/313	313/317	317/317	313	317
Hypertensives	77	55	15	7	125	29
		(0.71)	(0.20)	(0.09)	(0.81)	(0.19)
Normotensives	76	64	10	2	138	14
		(0.84)	(0.13)	(0.03)	(0.91)	(0.09)

Chi-square analysis of total allele counts for the NOS2A polymorphism indicated a significant difference between the hypertensive and normotensive groups ($\chi 2 = 5.9$; $P = 0.016$, $df = 1$). The odds ratio for hypertension associated with the 4 bp insertion is 2.3 (317 bp versus 313 bp allele; 95% CI 1.1-4.8).

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